DOI: 10.1113/EP088972

TITLE Electrophysiological characterisation of atrial volume receptors using *ex-vivo* models of isolated rat cardiac atria

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This is an Accepted Article that has been peer-reviewed and approved for publication in Experimental Physiology, but has yet to undergo copy-editing and proof correction. Please cite this article as an Accepted Article; doi: 10.1113/EP088972.

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FUNDING INFORMATION

This work was supported by the University College Dublin School of Medicine and a BHF project grant (PG/14/53/309000, awarded to Dr. Susan Pyner).

RUNNING TITLE

Rat atrial mechanoreceptors

TOTAL WORD COUNT

7724 words

SUBJECT AREA

Neuroscience

NEW FINDINGS

What is the central question of this study?

What *ex-vivo* preparation of the rat's cavo-atrial junction is efficient for characterising atrial mechanoreceptors?

What is the main finding and its importance?

Type A, B and intermediate atrial mechanoreceptors were studied using four different *ex-vivo* preparations: static pressure, flow, open and euthermic. The optimal preparation (euthermic)

involved direct recording from the right cardiac vagal branch with a Langendorff style perfusion at 37°C. Type A receptors were most common, appeared insensitive to stretch and sensitive to atrial contraction. Type B and intermediate receptors were not isolated at 20°C but were observed closer to 37°C. The findings may suggest that type A and B receptors utilise different molecular transduction mechanisms.

ABSTRACT

Atrial volume receptors are a family of afferent neurons whose mechanically sensitive endings terminate in the atria, particularly at the cavo-atrial junctions. These mechanosensors form the afferent limb of an atrial volume receptor reflex which regulates plasma volume. The prevailing functional classification of atrial receptors arose as a result of *in-vivo* recordings in the cat and dog and were classified as type A, B or intermediate according to the timing of peak discharge during the cardiac cycle. In contrast, there have been far fewer studies of the common small laboratory mammals such as the rat.

Using several *ex-vivo* rat cavo-atrial preparations, a total of 30 successful single cavo-atrial mechanosensory recordings were obtained. These experiments show that the rat possesses type A, B and intermediate atrial mechanoreceptors as described for larger mammals. Recording these cavo-atrial receptors proved challenging from the main vagus but direct recording from the cardiac vagal branch greatly increased the yield of mechanically sensitive single units. In contrast to type A units, type B atrial mechanoreceptor activity was never observed at room temperature but required elevation of temperature to a more physiological range in order to be detected. The adequate stimulus for these receptors remains unclear however, type A atrial receptors appear insensitive to direct atrial stretch when applied using a programmable positioner. The findings suggest that type A and type B atrial receptors utilise different molecular transduction mechanisms.

KEYWORDS

atrial mechanoreceptors, atrial volume receptors, mechanotransduction, autonomic nervous system, plasma volume regulation, *ex-vivo* models

INTRODUCTION

Mechanotransductive nerve fibres play a critical role in the immediate and coordinated response to physical deformation. Well known mechanoreceptors include: muscle spindles and Golgi tendon organs which encode for proprioception and kinaesthesia (Jami, 1992; Matthews, 1972), Pacinian and Meissner's corpuscles which encode touch and friction (Bell, Bolanowski, & Holmes, 1994; Johnson, 2001; Loewenstein & Skalak, 1966) and Merkel cells which are primarily concentrated on volar digital skin and provide a sense of shape and texture (Johnson, 2001; Polakovicova, Seidenberg, Mikusova, Polak, & Pospisilova, 2011). Less well studied mechanosensors are internal and visceral and include plasma volume receptors whose sensory endings are primarily concentrated at the cavo-atrial and pulmonary-atrial junctions of the heart (Cheng, Powley, Schwaber, & Doyle III, 1997; Coleridge, Hemingway, Holmes, & Linden, 1957; Garcia, Cantin, & Thibault, 1987; Hainsworth, 1991; Holmes, 1957; Linden & Kappagoda, 1982; Tranum-Jensen, 1975; Woollard, 1926). When the atria are stretched during plethoric blood conditions, atrial volume receptors trigger neural and hormonal responses that promote fluid expulsion (through diuresis). Impulses from atrial volume receptors are conveyed from the atria via small cardiac branches of the vagal nerves (Berthoud & Neuhuber, 2000) to the nucleus of the tractus solitarius in the medulla (Spyer, 1994). Thereafter, action potentials are either integrated centrally or relayed to GABAergic neurons in the perinuclear zone of the hypothalamus (Grindstaff & Cunningham, 2001).

Neurons in the perinuclear zone then project to magnocellular neurons of the supraoptic and paraventricular nuclei, tonically inhibiting the release of anti-diuretic hormone from the neurohypophysis (Affleck, Coote, & Pyner, 2012; Hines, Toney, & Mifflin, 1994; Roland & Sawchenko, 1993; Tribollet, Armstrong, Dubois-Dauphin, & Dreifuss, 1985). Atrial stretch is also associated with decreased renal sympathetic nerve activity (Brennan Jr, Malvin, Jochim, & Roberts, 1971; Karim, Kidd, Malpus, & Penna, 1972; Linden, 1979). Decreased renal sympathetic nerve activity promotes diuresis by causing vasodilation of the glomerular afferent arteriole, increased glomerular filtration rate, reduced renin secretion from the juxtaglomerular apparatus and reduced salt resorption from the distal convoluted tubule (Burnstock & Loesch, 2017).

Characteristics of atrial volume receptors

The current electrophysiological classification of mechanically sensitive atrial receptors arose as a result of *in-vivo* investigations performed in the cat (Paintal, 1953, 1963). Atrial receptors are classified as type A, type B or intermediate depending on their peak discharge during the cardiac cycle. Type A receptors discharge during the atrial a wave (i.e. during atrial systole), type B receptors discharge during atrial filling and intermediate receptors discharge during both the atrial a wave and filling phase. *In-vivo* functional recordings of atrial volume receptors in the great veins and atria have been performed in dogs, cats, rabbits, monkeys and frogs (Amann & Schaefer, 1943; Chapman & Pearce, 1959; Coleridge et al., 1957; Dickinson, 1950; Jarisch & Zotterman, 1948; Neil & Zotterman, 1950; Walsh, 1947; Walsh & Whitteridge, 1945; Whitteridge, 1948). The ratio of type A to type B receptors observed during *in-vivo* investigations varies by species. In the cat the ratio is approximately 1:1 (Arndt, Brambring, Hindorf, & Röhnelt, 1971b; Langrehr, 1960; Paintal, 1963). In the dog there appears to be a preponderance of type B receptors with reported ratios of 3:14, 2:28 and 3:47 (Coleridge et al., 1957; Langrehr, 1960; Paintal, 1972). In one similar study in the monkey, the ratio of type A to type B atrial receptors was 1:9 (Chapman & Pearce, 1959). Intermediate receptors appear to be much less common than type A and B receptors. Arndt et al. (1971b) reported a single intermediate receptor and similarly Paintal (1963) reported that in the cat there were very few intermediate receptors. The exception to this observation is the rabbit where intermediate receptors appear to predominate. In one study which reported findings from nine receptors in the rabbit, two were type A, one was type B and six were intermediate (Kappagoda, Linden, & Mary, 1977).

Although a common laboratory animal model, there is a paucity of literature pertaining to the rat. This may reflect the technical challenges that arise when working with very fine anatomical structures. The first functional description of atrial volume receptors in the rat involved *in-vivo* cervical vagal recordings where atrial receptors were classified as possessing low (<25Hz) or high (>25Hz) frequency discharge (Thorén, Noresson, & Ricksten, 1979). In their discussion Thorén et al. (1979) reported that some fibres possessed cardiac rhythmicity and discharged during the atrial a wave or during atrial filling similar to Paintal's classification. Of sixteen units reported, ten were type B, two were intermediate and four had irregular firing patterns. Low and high frequency mechanically sensitive atrial receptors have

also been reported by other groups performing *in-vivo* investigations in the rat (Storey & Kaufman, 2004). Storey and Kaufman (2004) classified receptors again as high frequency (>30Hz) or low frequency (<30Hz) but did not elaborate as to whether these receptors correlated with Paintal's functional classification. In a hybrid *in-vivo/in-vitro* study on the rat cardiac vagus (O'Leary & Jones, 2003), thirty-six single unit recordings had cardiac rhythmicity and seven units had mixed cardiac and respiratory rhythmicity. However, a description of how these fibres related to Paintal's classification of atrial volume receptors was not included because their receptive fields were not specifically localised and hence these units may have been of atrial or ventricular origin.

In-vivo and *ex-vivo* models of atrial mechanoreceptors

Historically, combinations of fluid administration and / or balloon expansion have been used to elicit atrial volume receptor stimulation during *in-vivo* investigations (Bainbridge, 1915; Kappagoda, Linden, & Snow, 1972a, 1972b; Ledsome & Linden, 1964). Alternate approaches involve the creation of an isolated atrial pouch (Ledsome & Linden, 1967). An inherent limitation of *in-vivo* approaches is that it is not possible to fully exclude the confounding effects of other organ systems. *Ex-vivo* investigations into atrial receptor mechanotransduction are much less common than *in-vivo* studies. In one such study, strips of cat atrial tissue were subjected to sinusoidal stretch whilst vagal afferent discharge was recorded (Arndt, Brambring, Hindorf, & Röhnelt, 1974). Vagal discharge patterns consistent with Paintal's classification were not observed and this led Arndt et al. (1974) to propose that only a single type of mechanically sensitive atrial receptor existed and that type A or type B discharge patterns could be explained by the anatomical configuration of the receptor with cardiac muscle. Chapman and Pankhurst (1976) (Chapman & Pankhurst, 1976) prepared isolated preparations of right atria and subsequently performed balloon distension at the cavo-atrial junctions. Thereafter, they opened the atrium and performed localised endocardial stretching with fine dissecting forceps. They achieved prolonged receptor viability that could be maintained for hours with coronary perfusion (Langendorff, 1895) and noted directional sensitivity for stretch in four of five receptors. Mifflin & Kunze (1982, 1984) investigated mechanically sensitive discharge from *ex-vivo* preparations of rat left cranial vena cava (Mifflin & Kunze, 1982, 1984). Preparations underwent stepwise and ramping changes in pressure and in their reports, Mifflin and Kunze describe two groups of receptors based on

their adaptation properties (slowly or rapidly adapting) and a very strong linear relationship between frequency of discharge and strain. Most reports of atrial receptor function are based on *in-vivo* investigations and only a small number of *ex-vivo* investigations have also been described. *Ex-vivo* recordings of atrial mechanoreceptor discharge that conforms to Paintal's classification (Paintal, 1963) have not been conducted hitherto.

Molecular basis of mechanotransduction

The precise molecular mechanism of mechanotransduction remains unknown however various molecular targets have been implicated. Pharmacological antagonism of the epithelial sodium channel inhibits the muscle spindle's ability to encode static stretch (Simon, Shenton, Hunter, Banks, & Bewick, 2010). Recycling of synaptic-like vesicles and the application of exogenous glutamate have been shown to enhance the muscle spindle's ability to encode stretch (Banks, Bewick, Reid, & Richardson, 2002; Bewick, Reid, Richardson, & Banks, 2005). Immunofluorescence studies have localised vesicular glutamate transporter 1 to the mechanosensitive endings of the muscle spindle and Golgi tendon organ (de Nooij et al., 2015). Transient receptor potential (TRP) channels constitute a large family of polymodal receptors. Immunofluorescence investigations have shown that TRPC5 is expressed in the baroreceptive endings of axons innervating the aortic arch and patch clamp studies of primary cultured rat aortic baroreceptor neurons have shown that TRPC5 channels are activated dramatically when cells are subject to micropipette pressure (Lau et al., 2016). Furthermore, in TRPC5 knockout mice, pressure induced afferent discharge from aortic baroreceptors is reduced and baroreflex mediated heart rate reduction is also attenuated (Lau et al., 2016). Immunohistochemical techniques have shown that TRPC1 and TRPV4 co-label with synaptophysin fibres in the atrial endocardium (Shenton & Pyner, 2014). It is interesting to note that there is already evidence that TRPV4 antagonism both prevents and resolves heart failure related pulmonary oedema (Thorneloe et al., 2012). It not unreasonable to suggest that TRPC5 and / or TRPV4 could contribute to atrial mechanotransduction. Piezo 1 and 2 channels have been implicated in the regulation of blood pressure and the baroreceptor reflex (Zeng et al., 2018). Compared to wild type, double knockout (Piezo 1 and Piezo 2) mice have greatly attenuated aortic depressor nerve activity following phenylephrine infusion. In the same study, double knockout mice possessed significantly higher mean arterial pressure, greater maximum blood pressure and lower minimum blood pressure values than their wild

type counterparts indicating reduced sensitivity of the baroreceptor reflex. Piezo could contribute to atrial mechanotransduction but to date this has not been investigated. Establishing an *ex-vivo* model of atrial mechanotransduction would permit pharmacological investigations into the molecular mechanisms that regulate plasma volume. Better understanding of these mechanisms could lead to novel therapeutic approaches for diseases such as congestive cardiac failure and hypertension.

Aims and hypotheses

We hypothesised that type A, B and intermediate atrial receptor discharge can be characterised utilising *ex-vivo* models and we aimed to utilise heart-vagal nerve preparations of the rat right atrium to verify this hypothesis. In addition, we aimed to establish an efficient *ex-vivo* heart-vagal nerve model that would facilitate pharmacological investigation into the molecular mechanism(s) of atrial mechano-sensation. To this end several preparations are described and one optimal approach is recommended.

METHODS

Ethical approval

The experimental work was carried out at University College Dublin in accordance with EU directive 2010/63 and local ethical committee approval (AREC-15-36-Jones). It was conducted in accordance with the principles enunciated in the "Guide to care and use of laboratory animals" (NIH 8th Edition, 2011). All animals had free access to food and drink and were purchased from University College Dublin Biomedical Facility or Charles River Laboratories (UK). The large number of animals used in this study can be further justified by stating that the tissues were also used for neuropharmacological and immunohistochemical investigations which will be the subject of future publications in this field of study.

Preparation of solutions and recording apparatus

Fresh HEPES buffered (10mM, Sigma-Aldrich, H7006) Tyrode's solution was prepared, bubbled (100% O_2) and pH adjusted to 7.4 with 1M hydrochloric acid using a calibrated pH meter. Heparin saline solution was prepared at a concentration of ≥500 I.U./ml (Acros Organics, 41121). Nerve recordings were performed using a glass suction electrode [fabricated from glass capillary tubes (Harvard Apparatus G150-4, Narishige PC-10)], acrylic electrode holder (Harvard Apparatus, ESW-M15P) and Neurolog system (Digitimer Ltd). The Neurolog system consisted of an NL100AKS Headstage, NL100C Stimulus Controller, NL104 A.C. Preamplifier and NL125 Filter. Output from the Neurolog system was fed to a 1401 MKII microcontroller (CED) and data were recorded with a laptop and Spike2 (version 8.01c) software (CED). For the initial experiments (static pressure and flow models of the right atrium), the pressure transducer (Spectramed Statham, P23XL) was calibrated using a mercury sphygmomanometer (Accoson, Dekamet). For subsequent experiments, a novel XYZ positioning system was utilised (Campbell & Jones, 2020) and the force transducer (FT03, Grass Instruments) was calibrated using a 10mN weight before each experiment. Pressure and force transducer output was amplified by a Grass Instruments CP122 A.C./D.C. strain gage amplifier.

Euthanasia and post-mortem surgery

All animals were euthanised by cervical dislocation after being rendered insensate with 5% isoflurane anaesthesia (in oxygen 5L / min). Using large dissecting scissors, a median sternotomy was performed. The thoracic cage was opened anteriorly and heparin saline (2ml, ≥500 I.U./ml) was injected into the cavity of the right ventricle. The exposed tissue was briefly perfused with oxygenated Tyrode's solution at room temperature. The lungs and thymus were carefully removed without causing trauma to the right atrium, inferior vena cava (IVC) or the neurovascular structures of the superior mediastinum. The IVC, descending aorta and oesophagus were cut at their diaphragmatic border. Careful incisions were made along the anterior margin of the vertebral bodies in order to mobilise the mediastinal bloc. A transverse incision was made across the trachea and great vessels of the neck in order to mobilise the mediastinal bloc which was subsequently perfused in oxygenated Tyrode's solution. Thereafter, the right atrium, right vagal nerve and its cardiac branch were isolated and either "static pressure", "flow", "open", or "euthermic" models of the right atrium were prepared.

Static pressure model and analysis

Fifty-seven male Wistar rats (88-430g) were included in this portion of the study. The inferior vena cava was tied inferiorly with surgical thread (Fine Science Tools, 18020-60). The superior vena cava (SVC) was tied carefully at its most superior point, with care taken not to disrupt the right vagal nerve or its cardiac branch. The left cranial vena cava (LCVC) was tied with surgical thread. The tricuspid valve was cannulated with a double lumen catheter (outer tubing: 1.5mm OD & 1.2mm ID, inner tubing 0.8mm OD & 0.4mm ID). A surgical tie was made across the atrioventricular groove to secure the catheter. The inner lumen was connected to a pressure transducer whilst the outer lumen was connected to a vertical column of oxygenated Tyrode's solution (Figure 1A).

The course of the right vagal nerve was explored and its cardiac branch identified. To ensure that nerve recordings were of cardiac origin, all other non-cardiac branches of the right vagal nerve were cut prior to recording. Considerable care was taken to avoid causing trauma to the cardiac branch of the right vagal nerve which is exquisitely sensitive to stretch. A pair of fine dissecting forceps were used to desheath a portion of the proximal main vagal nerve and nerve recording was performed using a glass suction electrode. Many attempts were usually required to locate a mechanically sensitive fascicle. When these fascicles could not be located, a segment of proximal right main vagal nerve was removed and the sampling process was repeated. When no mechanically sensitive afferents could be isolated, direct recording from the cardiac branch of the vagus nerve was performed. All recordings were performed at 20°C.

Static pressure preparations were investigated under euvolemic conditions. The right atrium was pressurised by one of two vertical columns of oxygenated Tyrode's solution (Figure 1B). These Tyrode's columns were placed at differing heights with respect to the heart and were continuous with the lumen of the right atrium through a three-way stopcock valve. One column was level with the heart whist the other column was raised until the right atrium was euvolemic. The pressure imparted to the *ex-vivo* heart-vagal nerve preparation (static pressure or flow) was equal to the product of ρgh (density, acceleration due to gravity and height of Tyrode's column).

All candidate mechanically sensitive atrial receptors were discriminated and analysed using Spike2 software (version 8.01c, CED) and Prism (version 5.01, GraphPad Software Incorporated). Post-stimulus histograms of afferent discharge with respect to the atrial a wave were generated and overlaid using Inkscape (version 0.92). Interval phase histograms were generated where 0° to 360° represented a single atrial cycle as measured from one atrial pressure peak (0°) to the next (360°). Mean phase angle for each mechanoreceptor was calculated by measuring the phase difference between peak atrial pressure and peak mechanoreceptor discharge. The phase angle for each type of atrial mechanoreceptor was expressed as mean ± standard deviation.

Flow model

Forty-three male Wistar rats (105-768g) were included in this portion of the study. The IVC was cannulated with polythene tubing connected to a vertical column of oxygenated Tyrode's solution (Figure 2A). The LCVC was tied and the SVC was cannulated with polythene tubing (1.5mm OD, 1.2mm ID) which led to the pressure transducer. Outflow was permitted through the tricuspid valve. The ascending aorta was cannulated and perfused with oxygenated Tyrode's solution (Langendorff style perfusion). Surgical thread was used to secure the catheter in the ascending aorta. All recordings were performed at 20°C. Stimulus control, nerve recording strategies and analysis of mechanically sensitive units was identical to the static pressure model.

Open model

Ten female Wistar rats (168-251g) were included in this part of the study. After removal of the ventricles, the atria were oriented lumen-upwards and constantly bubbled with oxygenated Tyrode's solution (100% O_2, Figure 2B). The proximal SVC, proximal LCVC and distal IVC were pinned on silicone. The apex of the left auricle was pierced with a hook fashioned from a 22-gauge needle and connected to the force transducer using surgical thread. The apex of the right auricle was pierced with a hook fashioned from a 22-gauge needle and connected to the X-Carriage of the XYZ positioning system using a 3D-printed

adapter. The atria were subjected to combinations of dynamic and static stretch which were applied by the XYZ positioning system (Campbell & Jones, 2020). The appropriate stretch distance was assessed during each experiment and ranged from 3-10mm. Nerve recordings were performed directly from the cardiac branch of the right vagal nerve which was identified at the root of the superior vena cava.

To confirm that the isolated nerve was the cardiac branch of the right main vagal nerve, stimulation was applied whilst cardiac parameters were monitored. The stimulus parameters used were 30Hz stimulation frequency, 1ms stimulus duration, 10-30V amplitude and were applied using an SD9 Stimulator (Grass Instruments). Negative inotropy or chronotropy confirmed that the nerve was the cardiac branch of the vagal nerve. Afferent discharge was recorded using a glass suction electrode and recordings were taken from multiple fascicles of the cardiac branch until mechanically sensitive afferents were isolated. All recordings were performed at 20°C. For a unit to be considered a mechanically sensitive atrial receptor, it was required to fulfil one of two specific inclusion criteria. First, single units were required to be reproducibly mechanically sensitive. For the static pressure and flow models, this was assessed by using the three-way stopcock valve to toggle between different distension pressures. For the open model, this was assessed by using the XYZ positioning system to apply graded stretch to the atria. Second, single units were required to discharge at specific points in the atrial cycle. This was assessed by using Spike2 (CED) to generate post-stimulus histograms of afferent discharge with respect to either the atrial a wave or stretch applied by the XYZ positioning system. Histograms that possessed a flat profile were excluded whilst those with clear peaks occurring at regular intervals during the atrial cycle or in response to graded stretch were included.

Recording from short cardiac nerves close to the epicardial surface increased the magnitude of ECG artefact in the recordings. However, a comparison of the timing of discharge relative to averaged atrial force showed that type A atrial receptor discharge occurred during the mid to late atrial contraction while ECG immediately preceded every atrial mechanical event (Figure 6A). To differentiate further the ECG artefact spikes from axonal action potentials, a back-averaging technique using Spike2 (CED) software was implemented. This involved using the wavemark spikes as triggers for their own averaging. The result was a sharpening of

the waveform and increase of signal to noise ratio. Axonal action potentials were much briefer than atrial electrograms (Figure 6B).

Euthermic model

Four female Wistar rats were included in this portion of the study (244-255g). The SVC was tied and the IVC was cannulated with a double lumen catheter: one lumen was continuous with the Tyrode's columns whilst the other lumen was continuous with the pressure transducer. The ascending aorta was cannulated and the coronary circulation was perfused (Langendorff, 1895). Outflow was permitted freely through the tricuspid valve (Figure 8A). The preparation was maintained at 37°C within a jacketed tissue bath and temperature was monitored using a standard mercury thermometer placed in the tissue bath. Pressure was imparted to the right atrium by toggling a three way stop-cock valve. Glass suction recording electrodes were used to record directly from the cardiac branch of the right vagal nerve.

Spike waveform analysis

Atrial mechanoreceptor spike waveforms were analysed using Spike2 (CED). The number of phases per spike was noted. Mean spike height and mean spike width for atrial mechanoreceptor waveforms were measured using Spike2 (CED). Spike height was defined as peak voltage deflection versus baseline. Spike width was measured at half spike height. A table of results was generated to facilitate comparison of type A, B and intermediate spike waveforms (Table 2).

RESULTS

Recordings from static pressure and flow atrial models

From 100 experiments, 244 single units were isolated. One hundred and forty-two units were deemed to be cardiac. Twelve single units qualified as mechanically sensitive atrial receptors, an example of which is presented in Figure 4. Five atrial receptors were isolated using the static pressure model and seven were isolated using the flow model. Ten type A, one type B and one intermediate atrial receptor was identified (for examples see Figure 5). Type A

discharge patterns were observed in both the static pressure and flow models. The type B atrial receptor was isolated using the flow model. The intermediate atrial receptor was isolated using the static pressure model. The type B and intermediate receptors were isolated while each preparation was at approximately 30°C. The mean phase angle for type A atrial receptors was 58 ± 40° (SD). The phase angle for the type B atrial receptor was 148°. For the intermediate atrial receptor, phase angle was 50° and 260° for the first and second peak respectively. The haemodynamic properties of these heart-vagal nerve preparations were: atrial pressure 4.2 ± 1.7 mmHg and heart rate 86 ± 10 beats per minute (expressed as mean ± SD). In addition, it was observed that coronary perfusion (Langendorff, 1895) greatly improved the longevity of preparations (~2 hours for non-coronary perfused preparations, ~6 hours for coronary perfused preparations).

Observations from the open model

Stimulation of the cardiac branch of the right vagal nerve elicited negative chronotropy and inotropy (Figure 3). Stimulation decreased mean peak systolic force from 11.8 ± 1.3 to 9.7 ± 1.4 mN (mean ± SD; n = 4). Stimulation also decreased mean heart rate from 101.7 ± 9.3 to 70.6 ± 21.2 beats per minute (n = 4). These effects were reversed within 30 seconds of the cessation of stimulation. From eighty-eight single unit recordings, eight mechanically sensitive units were isolated. These eight mechanically sensitive units were responsive to atrial systole but not graded stretch as applied by the XYZ positioning system. The other eighty units did not respond to atrial contraction or graded stretch as applied by the XYZ positioning system. For each mechanically sensitive unit, peak discharge occurred during atrial systole similar to the type A discharge patterns observed with the static pressure and flow models (Figure 6A and 7A). The peak discharge for all eight type A atrial receptors occurred during the mid to late atrial a wave. No type B or intermediate receptors were observed. All eight mechanically sensitive units were stimulated by atrial systole and not by graded stretch as applied by the XYZ positioning system as evidenced by the histogram profiles presented in Figure 7.

Recordings from the euthermic model

Mechanically sensitive units were isolated in three out of four pilot experiments. In total, 42 single units were isolated: 10 units were mechanically sensitive and 32 were not. For all mechanically sensitive units, post-stimulus histograms and mean atrial pressure waveform averages were overlaid which showed that 6 were type A, 3 were type B and one was an intermediate atrial volume receptor (for examples see Figure 8B). While recording of these mechanically sensitive units, mean atrial pressure was 2.4 ± 2.1 mmHg and mean heart rate was 164 ± 54 beats per minute (mean ± SD; n = 7). Excellent preparation viability was noted despite the elevated temperature (37°C) versus static pressure, flow and open models (20°C).

Spike waveform analysis

Thirty atrial mechanoreceptor waveforms were analysed (24 type A, 4 type B and 2 intermediate). Most type A atrial mechanoreceptor waveforms were triphasic (n = 21) and a small number were biphasic (n = 3). All type B (n = 4) and intermediate (n = 2) waveforms were triphasic. During each phase, spike height and width were similar for all atrial mechanoreceptor subtypes (Table 2).

Model	Rats	Number of non-mechanically sensitive units	Number of mechanically sensitive units	% yield of mechanically sensitive units per rat	% Type A per model	% Type B per model	% Intermediate per model
Static Pressure	57	115	5	9	80	0	20
Flow	43	117	7	16	86	14	0
Open	10	80	8	80	100	0	0
Euthermic	4	32	10	250	60	30	10
Total	114	344	30				

Table 1 Yield efficiency of mechanically sensitive single units by model

	Phase 1				Phase 2				Phase 3			
	Spike Height (μV)		Spike Width (ms)		Spike Height (μV)		Spike Width (ms)		Spike Height (μV)		Spike Width (ms)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Type A (biphasic, n = 3)	27.53	22.70	0.50	0.10	22.41	21.84	0.79	0.26				
Type A (triphasic, n = 21)	17.24	10.65	0.44	0.13	27.70	8.51	0.48	0.16	6.97	2.40	0.93	0.32
Type B (all triphasic, n = 4)	17.48	4.38	0.26	0.11	24.25	5.37	0.36	0.09	7.27	2.54	0.69	0.23
Intermediate (all triphasic, n = 2)	26.75	32.88	0.39	0.25	23.85	19.45	0.41	0.11	3.85	1.05	0.71	0.45

Table 2 Spike waveform characteristics of atrial mechanoreceptors by type

Note that spike height and width were similar for all atrial mechanoreceptors (SD = standard deviation).

DISCUSSION

Recordings of atrial mechanoreceptors from static pressure and flow models

Mechanically sensitive atrial receptor discharge was successfully isolated using both static pressure and flow models. The overall yield of mechanically sensitive atrial receptor afferents was low. Both models yielded roughly equivalent numbers of cardiac units but the overall yield was low. Ten of the recorded units were type A, one was type B and one was intermediate. It should be noted that the type B and intermediate receptors isolated from our initial experiments were recorded at approximately 30°C whilst we assessed the viability of the preparation at temperatures closer to physiological range. This differs from the rest of the isolated atrial mechanoreceptors which were recorded at 20°C and were all type A. All units exhibited peak discharge at specific points in the atrial cycle (Figure 5). Mechanically sensitive atrial receptors were observed to discharge in a 1:1 ratio with the atrial cycle except for the intermediate unit which discharged twice per atrial cycle (Figure 5C). The observed discharge patterns conform with the classification described by Paintal (1963). In addition, all twelve mechanically sensitive units would be categorised as low frequency as described by Thorén et al. (1979) or Storey & Kaufmann (2004). Interestingly, we did not observe any instances of high frequency discharge from mechanically sensitive units. This may be attributable to our lower recording temperatures (20°C) and slower heart rates versus *in-vivo* models.

The classification of mechanically sensitive atrial receptor discharge only by frequency is limited as it does not take account of the cardiac cycle. Thorén et al. (1979) did not possess the hardware and software capabilities to perform precise single unit discrimination as we have done and thus may have reported the combined discharge of many similar units. It has long been known that the discharge of mechanically sensitive atrial receptors relates to the cardiac cycle, to the extent that initially it was proposed that these receptors may serve as a rate meter for the central nervous system (Arndt, Brambring, Hindorf, & Röhnelt, 1971a).

Attempts were made to localise the receptive fields for these receptors by using Von Frey fibres to probe the epicardial and endocardial surface. However, receptive fields could not be consistently located. Given the short length of axonal fibres and a lack of access to

subendocardial receptive fields, it was also not possible to trace the precise axonal length from recording electrode to receptor. As the conduction velocity for these axons could not be measured precisely, it was also not possible to determine the precise moment of receptor activation during the atrial cycle and thus each mechanoreceptor's activation threshold could not be precisely measured. Mifflin & Kunze (1982) also encountered this limitation during their investigations of the rat LCVC. It is hoped that this limitation can be overcome by adapting the XYZ positioning system (Campbell & Jones, 2020) to systematically probe the endocardial surface and thus map the receptive field of isolated atrial mechanoreceptors. Previous work on the rat has identified atrial mechanoreceptor axons as C-fibres (O'Leary & Jones, 2003; Thorén et al., 1979) with conduction velocities less than $2ms^{-1}$. O'Leary et al. (2003) utilised a double recording technique and spike triggered averaging to achieve this. The mean atrial pressure (4.2 mmHg) of the present study is in line with that reported in previous studies. Mifflin & Kunze (1984) reported that a pressure of 3.8 mmHg was in the range of threshold for most fibres and Thorén et al. (1979) similarly reported activation pressures of 2.5-9 mmHg. It is conceivable that our low yield of mechanically sensitive afferents was attributable to inadequate stimulation of the receptors but this does not seem like a satisfactory explanation considering that our *ex-vivo* models preserved the normal anatomical structure of the right atrium and were modelled as euvolemic preparations. In addition, our mean atrial pressures were within the normal physiological range for the great veins and right atrium.

The low yields of mechanically sensitive atrial receptors could be attributable to multiple factors. First, it is plausible that the cardiac branch of the vagal nerve or vagal nerve proper are susceptible to trauma during cervical dislocation though alternative methods of euthanasia are also associated with disadvantages. Most functional studies of the rat cardiac vagus have circumvented this problem by utilising *in-vivo* models (O'Leary & Jones, 2003; Storey & Kaufman, 2004; Thorén et al., 1979) but with these approaches there are confounding receptive fields in the respiratory system (e.g. bronchial mechanoreceptors can be stimulated by a beating heart). For our *ex-vivo* models, we did not attempt to model normal intrathoracic negative pressure as we presumed that this factor could be overcome by increasing the magnitude of the stimulus (i.e. raising the height of the Tyrode's column). Second, the neurosurgical portions of the surgery were technically challenging to perform. Whilst it was not technically challenging to desheath the right main vagal nerve or to record from it with a

suction microelectrode, it was time consuming to locate specific fascicles that convey mechanically sensitive afferent discharge. This necessitated repeated sampling and carried no guarantee of success. Fascicles conveying mechanically sensitive afferent discharge were rarely isolated immediately and typically would take 1-2 hours to isolate. When recording from the main vagal trunk, we opted to utilise wide-bore suction electrodes in order to cover a greater number of vagal fascicles and thus increase the probability of isolating a mechanically sensitive fascicle with each attempt. Third, due to the Law of LaPlace (Basford, 2002; Sugishita, Iida, Ohtsuka, & Yamaguchi, 1994), the adequate stimulus to elicit discharge from mechanically sensitive atrial receptors is a function of pressure, wall stress and chamber radius. The effect of each of these interdependent variables is challenging to isolate. Type A and type B mechanically sensitive atrial receptors may possess different discharge patterns because they are selectively sensitive to compression (type A) or stretch (type B) during the atrial cycle (as Golgi tendon organs and muscle spindles are in skeletal muscle). It is plausible that the application of a receptor-specific stimulus could increase the yields of atrial receptor recordings from our static pressure and flow models. In previous reports, many different stimulus application techniques have been utilised to elicit discharge from atrial receptors such as infusions of blood or saline (Bainbridge, 1915), balloon inflation (Kappagoda et al., 1972a, 1972b) or the formation of atrial pouches (Ledsome & Linden, 1967). It is important to note that in an *in-vitro* preparation of rat LCVC, Mifflin and Kunze (1982) demonstrated a linear relationship between strain and discharge frequency for mechanically sensitive receptors and it is this report which inspired an application of the XYZ positioning system to apply graded stretch to the open model of the atria.

Open model: stimulation and recording

The observed bradycardia and reduction in atrial systolic force is in accordance with previous investigations that reported vagally mediated negative chronotropic and inotropic effects (Jones, O'Leary, & Pickering, 2003). The observed effects indicate that the cardiac branch of the right vagal nerve had been identified correctly through anatomical means and that the cardiac ganglia remained viable. This branch was also traced directly to cardiac ganglia at the posterior aspect of the right atrium. The projection of cardiac vagal preganglionic neurons to this branch has been studied previously by anterograde labelling from the medulla (Izzo & Jones, 1994). All eight mechanically sensitive units discharged during the mid to late atrial a wave. This was consistent with observations made from the static pressure and flow models. No type B or intermediate receptors were observed which may be due to insufficient sampling. An alternative hypothesis is that this is attributable to inherent bias in the experimental design. Paintal believed that type B receptor discharge was related to atrial filling (Paintal, 1972) and this model does not adequately reproduce atrial filling. However, this alternative hypothesis is not supported by observations from the static pressure and flow models where atrial filling was reproduced yet only one receptor was type B and one was intermediate. The observed preponderance of type A receptors differs from reports of past *in-vivo* work in the rat where the majority of mechanically sensitive atrial receptors were type B receptors (Thorén et al., 1979). Thorén et al. (1979) reported that from sixteen recordings, ten were from type B receptors, two were from intermediate receptors and four had erratic discharge patterns. These conflicting observations may be attributable to the anatomical differences between these experimental approaches. It must also be considered that these approaches were performed at different temperatures. The investigation of Thorén et al. (1979) was performed at 37°C whereas most of our investigations were performed at 20°C. It is not unreasonable to suggest that different molecular mechanisms may be responsible for type A and type B atrial mechanotransduction. TRP channels are known to be sensitive to temperature (Inoue, Jian, & Kawarabayashi, 2009) and if TRP channels also contribute to type B and intermediate atrial receptor activity then our lower recording temperatures may also explain the absence of these receptor types at room temperature.

Combinations of dynamic and static stretch were performed by the XYZ positioning system though these approaches consistently failed to elicit discharge from mechanically sensitive

atrial receptors (Figure 7B). In addition, manual stretching of the right or left auricle, left cranial vena cava and the mid atrial region also failed to elicit afferent discharge. This was unexpected considering that previous *ex-vivo* work in the atria of the rat, there are reports of a linear relationship between strain and frequency of discharge (Mifflin & Kunze, 1982, 1984). In addition, simply stretching the endocardium of the cat using forceps has also been shown to be an effective stimulus to elicit atrial receptor discharge (Chapman & Pankhurst, 1976). It is possible that the region of stretch did not overlap with the receptive field of an atrial mechanoreceptor though this explanation does not seem adequate considering the varied way in which stretch was applied. Superfused models that preserve normal anatomical relations possess the inherent advantage that pressure and stretch are applied to the entire endocardial surface of the atrium.

This model was technically less challenging to prepare than the static pressure or flow models. In addition, the yield of recordings from mechanically sensitive atrial receptors was superior to the original preparations. The change from male to female rats was not implemented because of a specific scientific inquiry and was attributable to the researchers' need to balance the number of male and female animals used during the project. Eight atrial volume receptors were isolated from ten open model preparations whereas twelve atrial volume receptors were isolated from one hundred static pressure / flow model preparations. This improvement is likely attributable to changes in the nerve recording strategy. Mechanically sensitive afferents were isolated more efficiently when recording directly from the cardiac branch because the right main vagal nerve contains a large number of fibres that innervate other viscera. Mechanically sensitive afferents were typically isolated within 15 minutes when recording directly from the cardiac branch of the right vagal nerve.

Electrophysiological recordings at room temperature and body temperature

Nerve recordings for the static pressure, flow and open models were performed at 20°C rather than 37°C for three reasons. First, in the initial atrial experiments, it was observed that tissue viability was greatly reduced at temperatures above 28°C. Isolating the fascicle of a mechanically sensitive neuron from the cut section of the right main vagus was unpredictable and could take up to an hour. Thus, preparations were kept at room temperature to preserve tissue vitality and allow for additional time for mechanically sensitive atrial receptors to be isolated and studied. Langendorff perfusion was not utilised in the atrial static pressure models but was incorporated in the flow model where it was observed to greatly improve tissue vitality. Second, the rat heart exhibits a negative Treppe phenomenon whereby rising heart rates decrease inotropy (Figure 9A) rather than increasing it as seen in other species (Nanasi et al., 1996). Given that increased temperature is associated with increased heart rate, recordings were performed at room temperature in order to maximise inotropy and in turn it was speculated that this would increase the yield of type A mechanically sensitive atrial receptors. Third, we aimed to closely emulate the pharmacological experiments which identified the epithelial sodium channel as an important contributor to muscle spindle mechanotransduction (Simon et al., 2010). Given that these experiments were performed at 20°C, we also performed our experiments at 20°C to facilitate comparison. This pharmacological data will be the subject of a future manuscript.

The basal heart rate of the isolated rat heart at 37°C was 164 beats per minute which is comparable to previous reports [188 beats per minute, Jones et al. (2003)] but lower than *in-vivo* rates. The successful isolation of type A, B and intermediate receptors from the euthermic model suggests that the results obtained at 20°C are translatable to normal physiological temperature. Mechanically sensitive units were obtained in three of four euthermic pilot experiments similar to the open model (Table 1) and we attribute this to their shared nerve recording strategy. The high yield of single unit recordings (42 total) from the euthermic model experiments is indicative of excellent tissue viability. Of 10 mechanically sensitive units, 6 were type A, 3 were type B and one was an intermediate unit. With respect to recordings made at 20°C, there was an increased proportion of type B units isolated from the euthermic model. This could indicate that type B atrial volume receptors rely upon polymodal ion channels such as TRP channels which are known to be enabled by temperature

(Padinjat & Andrews, 2004; Ramsey, Delling, & Clapham, 2006). In further support of this hypothesis, one type B unit was recorded which was quiescent at 20°C but discharged with increasing frequency as the atrial preparation was heated (Figure 9A). The rise in temperature was associated with an increase in heart rate but a decline in mean atrial pressure as the rat heart exhibits a striking negative Treppe phenomenon (Nanasi et al., 1996). The high yield of mechanically sensitive units, varied distribution of atrial volume receptors and excellent tissue viability indicate that the euthermic model with a Langendorff perfusion system is the preferred model for future pharmacological investigations.

Interconversion of atrial mechanoreceptors

In-vivo recordings in the cat demonstrated that type A, B and intermediate atrial mechanoreceptors can be interconverted from one to another (Kappagoda, Linden, & Mary, 1976). Type B and intermediate receptors were converted to type A by controlled haemorrhage and administration of intravenous adrenaline. Type A receptors could be converted to type B and intermediate receptors by volume expansion. Kappagoda et al. (1976) proposed that this conversion phenomenon indicated that there is a single type of atrial mechanoreceptor and that discharge pattern merely reflects a spatial relationship (in series or in parallel) to myocytes. An alternative hypothesis was offered by Cheng et al. (1997) whose anterograde labelling of the rat endocardium demonstrated that vagal afferent fibres possess three end-organs: end-nets, flower-spray endings and intramuscular endings. Cheng et al. (1997) proposed that type A discharge arises from intramuscular endings, type B from flower-spray endings and that intermediate discharge arises from polymorphic fibres that possess both intramuscular and flower-spray endings. However, Cheng et al. (1997) were unable to reconcile their observations with those of Kappagoda et al. (1976). Our hypothesis that type A and B atrial mechanoreceptors may possess different molecular transducers is compatible with that of Cheng et al. (1997) but incompatible with that of Kappagoda et al. (1976). In the present study, conversion of type A to B with volume expansion was not observed but this may be due to the fact that most experiments were conducted at room temperature where type B activity was absent. However, at 37°C, two of six type A units exhibited phase shift while under volume expansion. It was not possible to determine the direction of the shift though in both cases peak receptor discharge still occurred during atrial contraction and not during atrial diastole.

Conclusions

Type A, B and intermediate atrial mechanoreceptors were found in the rat right atrium. In contrast to type A units, type B atrial mechanoreceptor activity was only observed above room temperature. The adequate stimulus for these receptors remains unclear, however type A atrial receptors appear most sensitive to atrial contraction. The viability and longevity of right atrial preparations can be extended with the inclusion of a Langendorff-style coronary perfusion. The optimal recording strategy is to sample from the cardiac branch of the right vagal nerve and not the vagal trunk. Pharmacological agents can be applied to these ex-vivo heart-vagal nerve preparations to elucidate the molecular basis of atrial mechanotransduction. Such investigations could lead to the discovery of novel therapeutic approaches in the management of congestive heart failure and hypertension.

AUTHOR CONTRIBUTIONS

The authors take responsibility for the integrity of the data and accuracy. The experimental work was conceived in a collaborative manner by all authors. The experimental work was carried out at University College Dublin by Dr. Thomas Campbell, Dr. Eric Lucking and Prof. James FX Jones. The results were analysed and interpreted by all authors. The manuscript was prepared by Dr. Thomas Campbell and overseen by Dr. Fiona Shenton, Dr. Eric Lucking, Dr. Susan Pyner and Prof. James FX Jones. All authors have reviewed the manuscript and consented to its submission.

COMPETING INTERESTS

None declared.

ACKNOWLEDGEMENTS

None declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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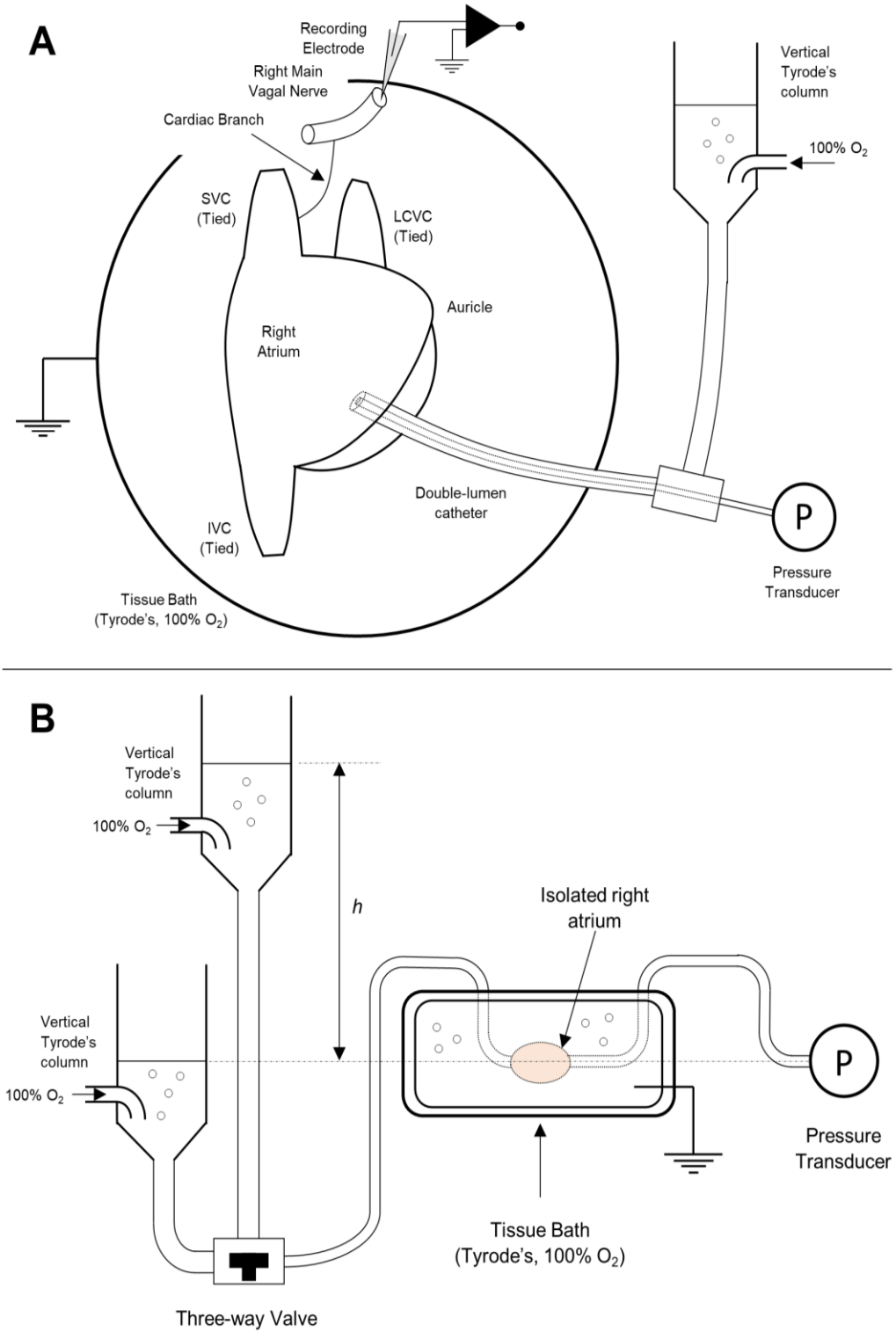

Figure 1: Static pressure model and stimulus control system. (A) Static Pressure Model. The double-lumen catheter was inserted through the tricuspid orifice. A surgical tie was made across the atrioventricular groove to secure the catheter. The great veins were tied. **(B)** Pressure control system for ex-vivo heart-vagal nerve models. This system was used to control pressure for both static pressure and flow models. The three-way valve was used to alternate which Tyrode's column was continuous with and thus imparted pressure to isolated preparations of the right atrium. Single unit nerve recordings were assessed for mechanical sensitivity by alternating the right atrium between low and high pressure states. IVC, Inferior Vena Cava. LCVC, Left Cranial Vena Cava. SVC, Superior Vena Cava.

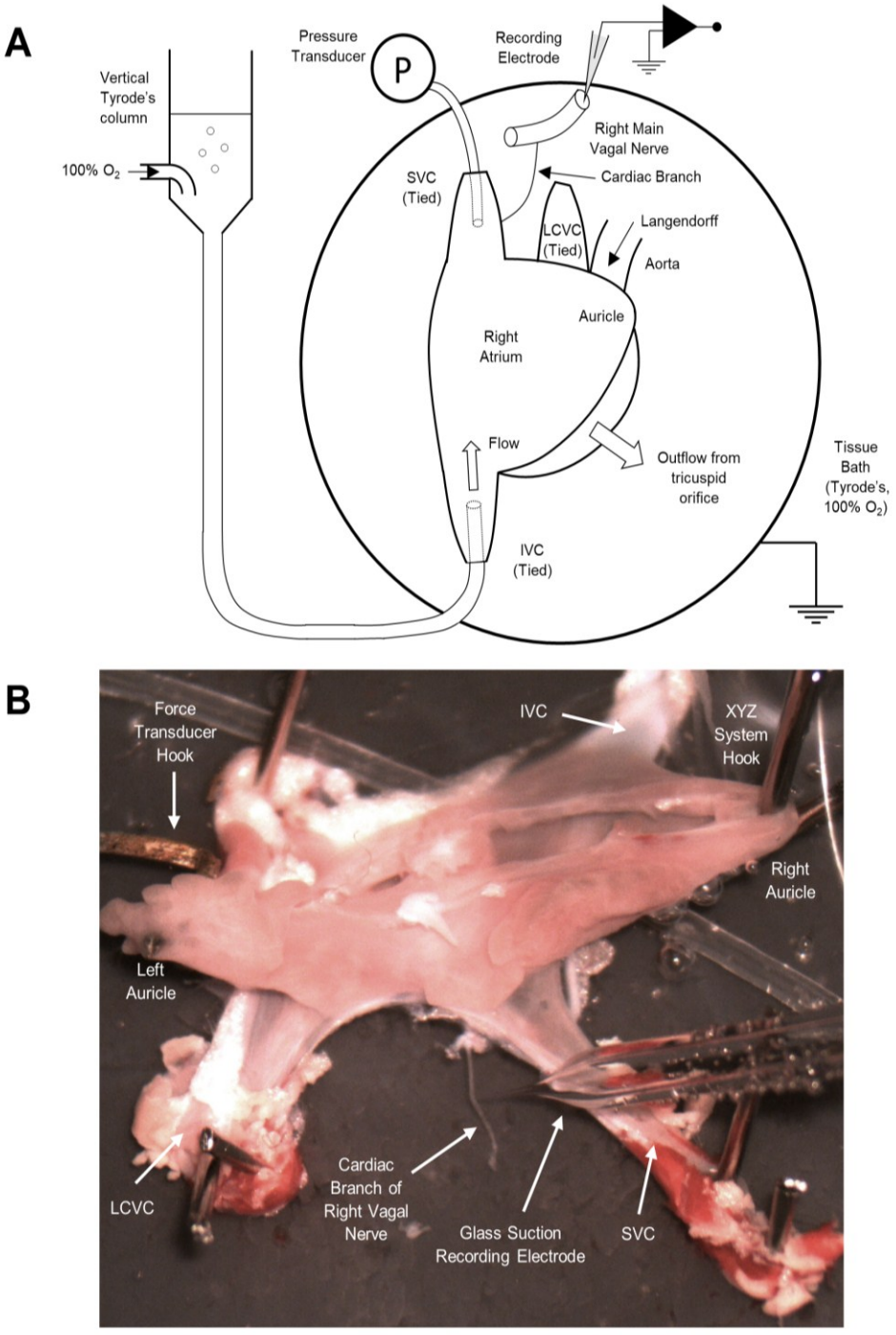

Figure 2: Flow model and open model. (A) Flow Model. The pressure transducer line was sited in the SVC and the Tyrode's column line was sited in the IVC. The LCVC was tied. **(B)** Open model. The atria of the rat were prepared as shown and perfused in HEPES buffered (10mM) Tyrode's solution (oxygenated with 100% O_2). The left auricle was connected to a force transducer (FT03, Grass Instruments) using surgical thread and a hook fashioned from a 22-gauge needle. The XYZ positioning system was connected to the right auricle using a 3D printed adapter and a 22-gauge needle fashioned as a hook. The XYZ positioning system was used to apply dynamic and static stretch to the atria whilst afferent spikes were recorded from the cardiac branch of the right vagal nerve. IVC, Inferior Vena Cava. LCVC, Left Cranial Vena Cava. SVC, Superior Vena Cava.

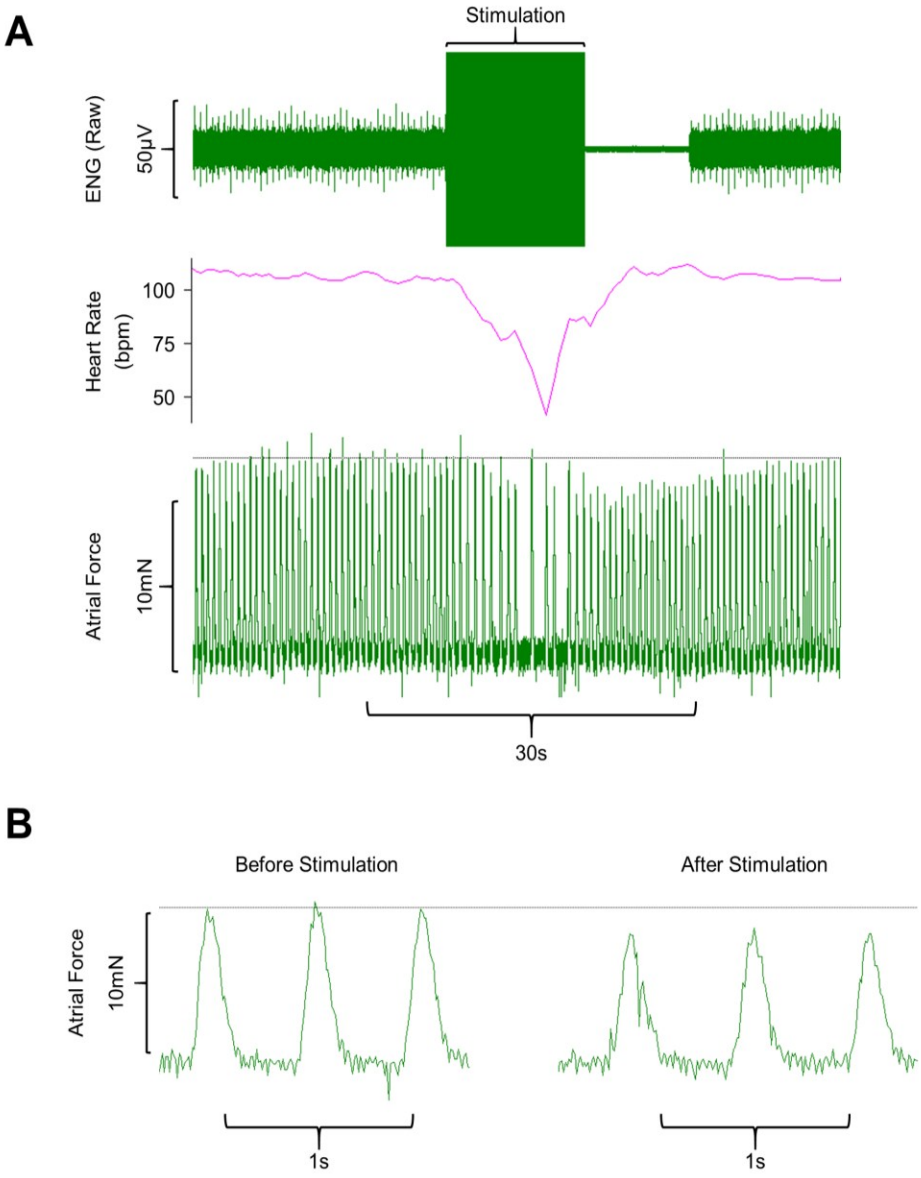

Figure 3: Stimulation of the right vagal cardiac branch elicits negative chronotropy and negative inotropy in the open model. (A) The cardiac branch of the right vagal nerve was identified anatomically and then stimulated (30Hz, 1ms stimulus duration, 30V). Stimulation elicited negative chronotropy and inotropy, confirming that the stimulated nerve was the cardiac branch of the right vagal nerve. **(B)** Three cycles of atrial contraction before and after stimulation showing that peak contractile force was reduced by stimulation. The observed negative inotropic effect confirmed that the stimulated branch was the cardiac vagal branch and thus subsequent recordings of afferent spikes were deemed to be of cardiac origin. Data recorded using Spike2 (CED) software. CED, Cambridge Electronic Design. ENG, electroneurogram.

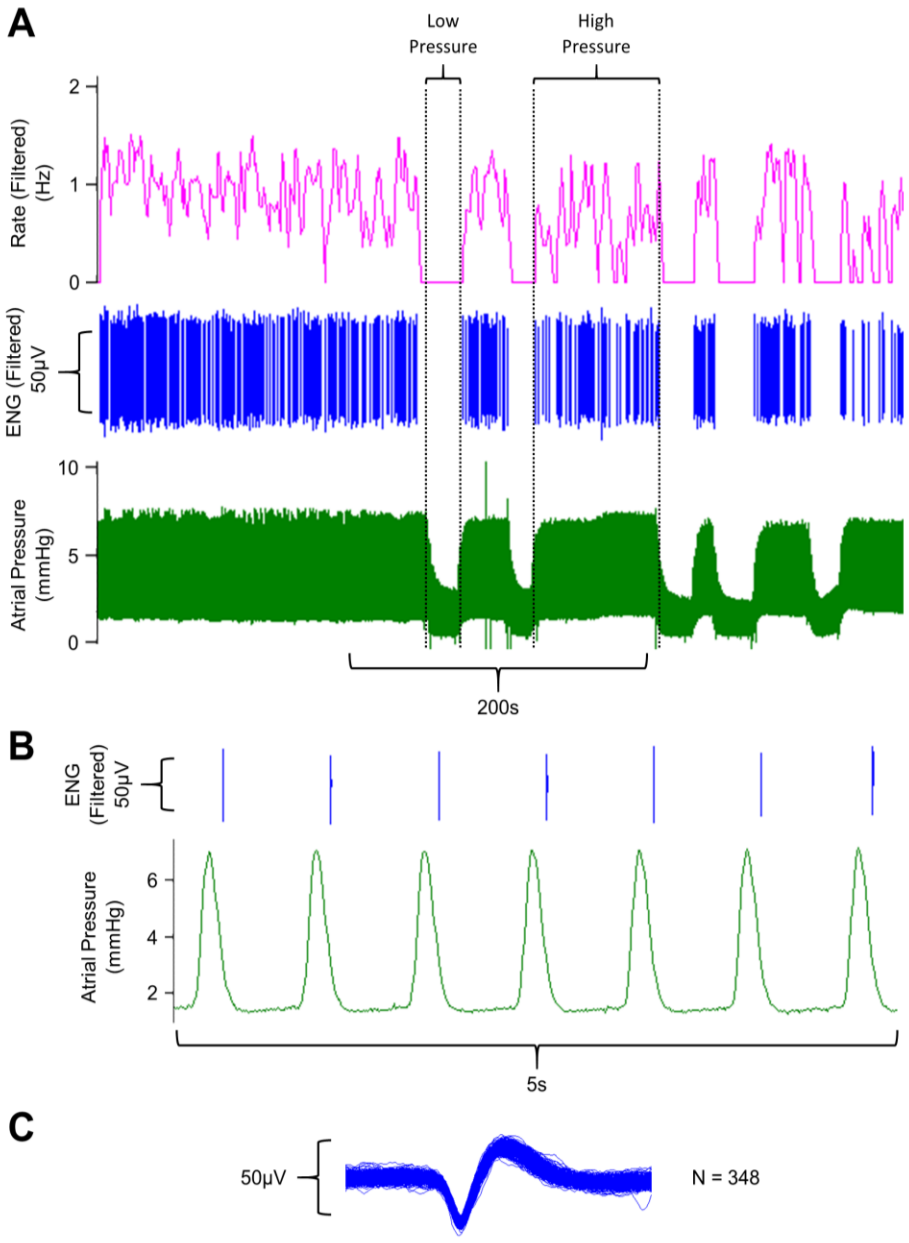

Figure 4: Pressure-discharge relationship for a type A mechanically sensitive atrial receptor isolated using the static pressure model. This unit was recorded during a static pressure preparation. **(A)** Raw trace from Spike2 (CED) showing atrial pressure (green), single unit vagal afferent discharge (blue) and discharge rate of single unit vagal afferent (pink). The right atrium was repeatedly cycled between low and high pressure states (green) in order to elicit discharge from mechanically sensitive atrial receptors. This unit fired during high pressure states and not during low pressure states. **(B)** Shown is a 5 second window of the high pressure state. As was typical for mechanically sensitive atrial receptors, this unit discharged at a specific point in the atrial cycle. **(C)** Overdraw (N=348) of filtered unit from (A) and (B) which was indicative of a single unit recording. CED, Cambridge Electronic Design. ENG, Electroneurogram.

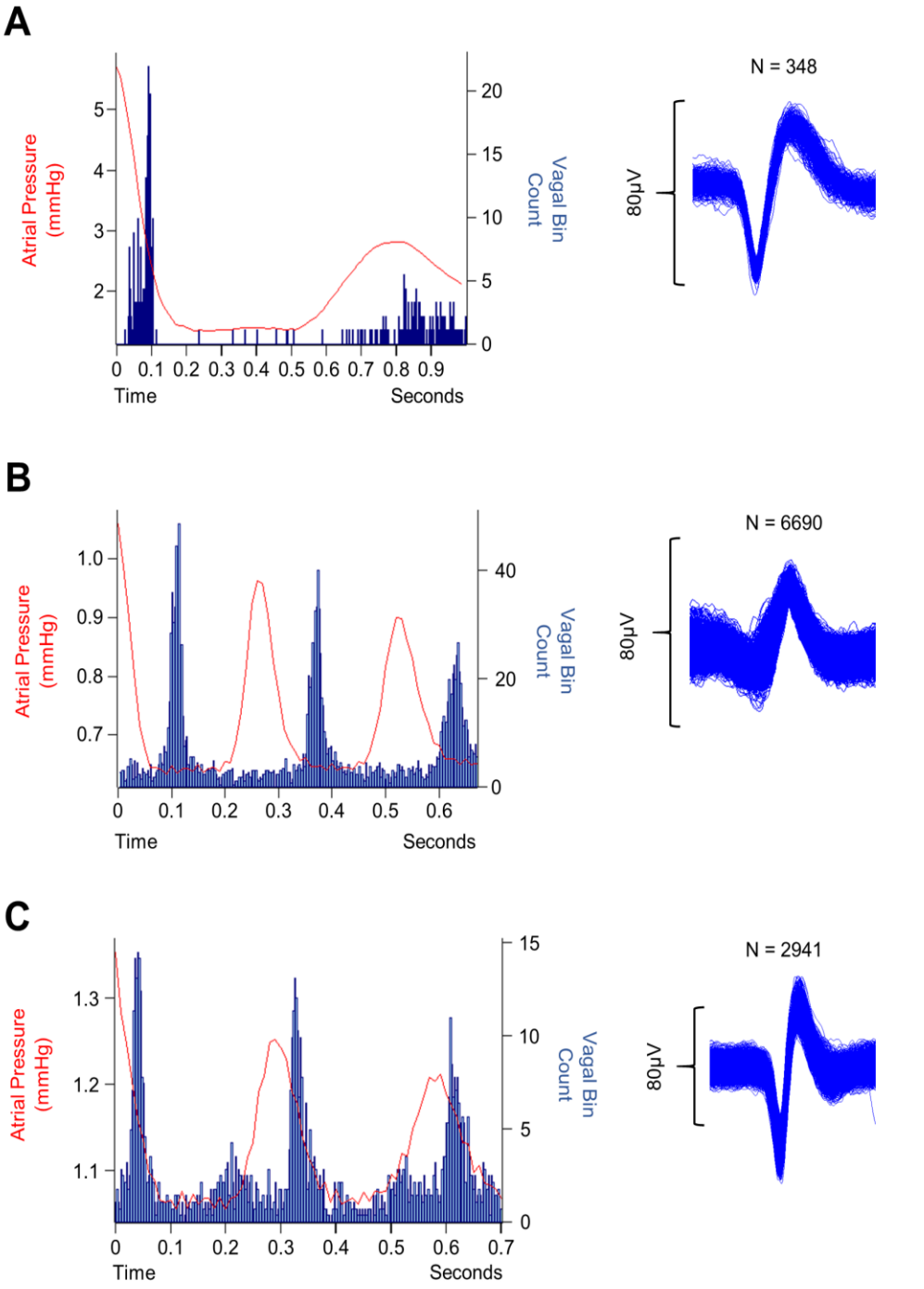

Figure 5: Type A, B and intermediate atrial receptors isolated using the static pressure model. (A-C) Shown (left) are overlays of average pressure waveform (red) and a post-stimulus histogram of vagal firing latency indicating the time in the atrial cycle at which these mechanically sensitive neurons discharge. Overlays were generated using Inkscape (v0.92). This data pertains to single unit recordings of atrial receptors and an overdraw of their respective spike waveform is presented (right). **(A)** Type A atrial receptor which discharges during the atrial a wave. **(B)** Type B atrial receptor which discharges during atrial filling. **(C)** An intermediate atrial receptor which discharges during both the atrial a wave and atrial filling. Increased heart rate was observed in both (B) and (C) as recordings were performed at approximately 30°C whilst (A) was recorded at 20°C.

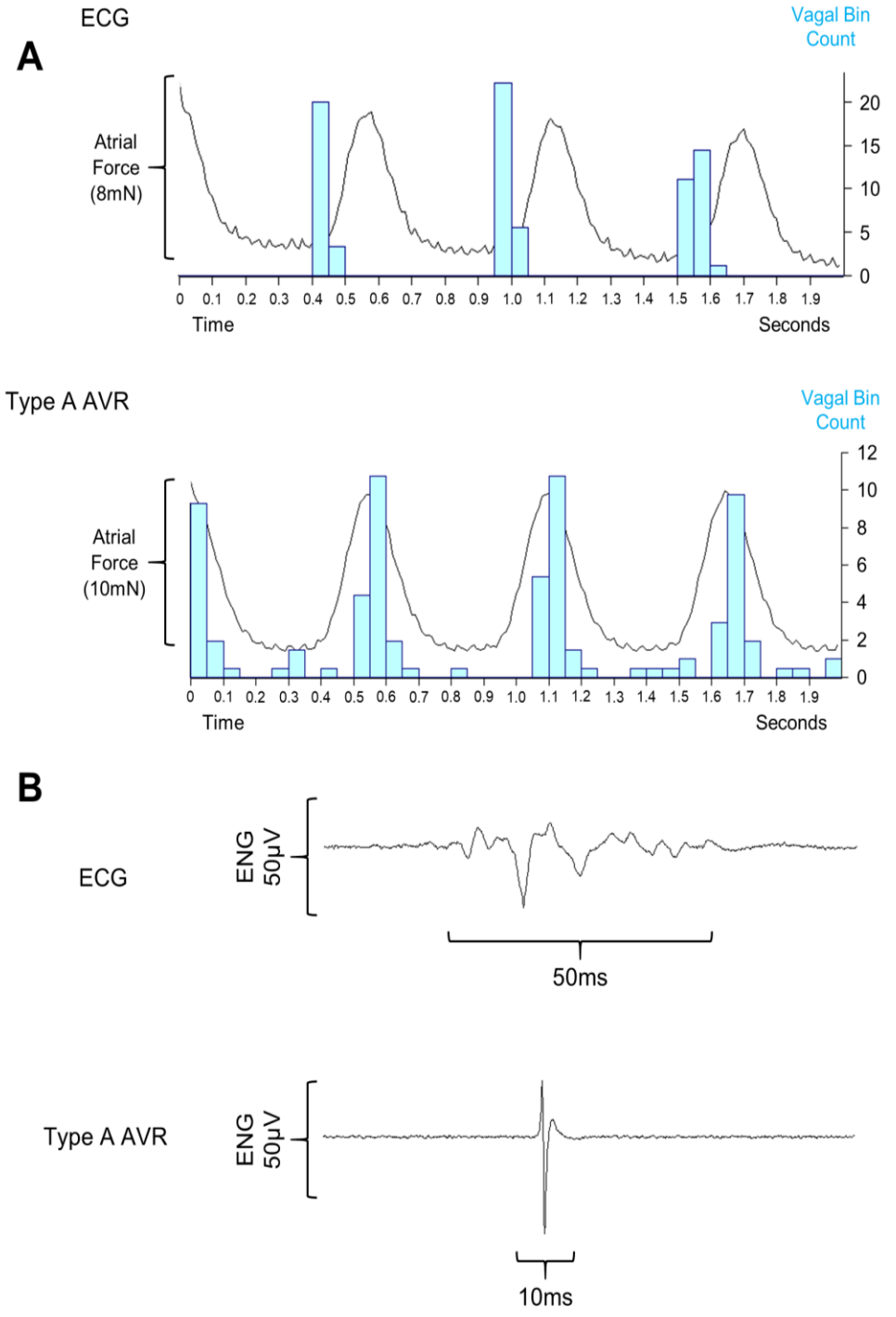

Figure 6: Discrimination between ECG and afferent spike activity for the open model. (A) Overlay of mean atrial force and histograms showing the relative distribution of ENG discharge during the cardiac cycle. Type A atrial receptors discharge during the atrial a wave whereas ECG precedes the atrial a wave. **(B)** Mean ENG waveform for ECG and a type A atrial receptor. The duration of the ECG waveform is much longer than that of the type A atrial receptor waveform. Data recorded using Spike2 (CED). AVR, Atrial Volume Receptor. CED, Cambridge Electronic Design. ECG, electrocardiogram. ENG, electroneurogram.

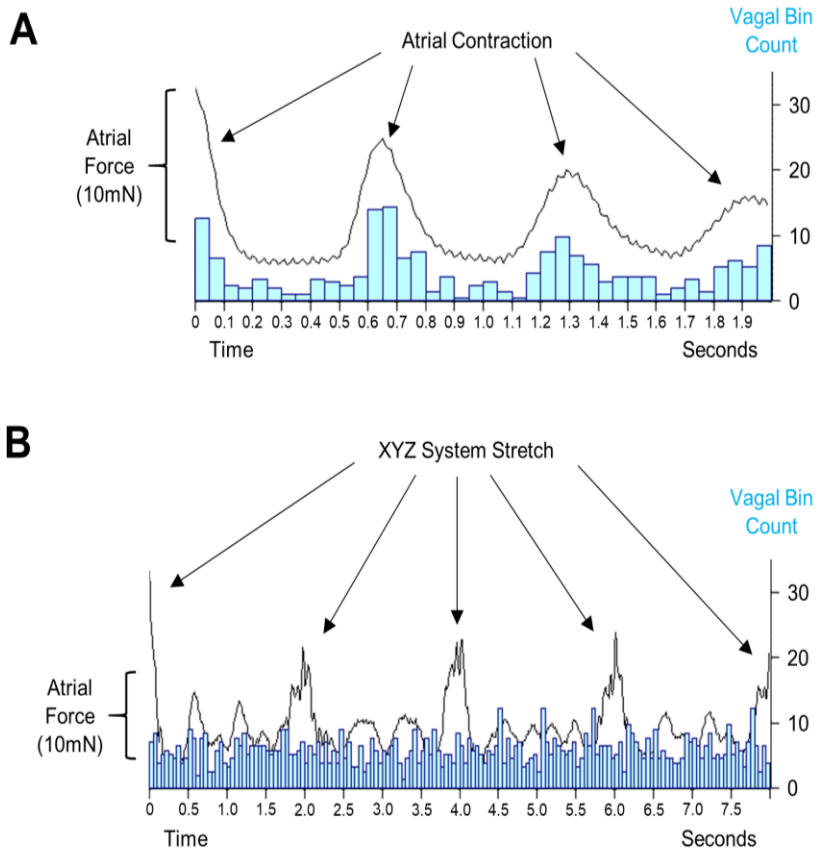

Figure 7: Graded stretch as applied by the XYZ positioning system does not elicit atrial receptor discharge from the open model. Average atrial force is overlaid with a histogram showing the distribution of atrial receptor discharge with respect to the atrial cycle or graded stretch applied by the XYZ positioning system. The data presented for (A) and (B) are taken from the same recording of a mechanically sensitive atrial receptor. Histogram bin width is 50ms for both (A) and (B). **(A)** Atrial receptor discharge versus atrial contraction. Peak discharge is observed during atrial systole which is characteristic of a type A receptor. **(B)** Atrial receptor discharge versus graded stretch as applied by the XYZ positioning system. The flat profile of the histogram indicates that graded applications did not elicit discharge from this atrial receptor. Data recorded using Spike2 (CED) software. CED, Cambridge Electronic Design.

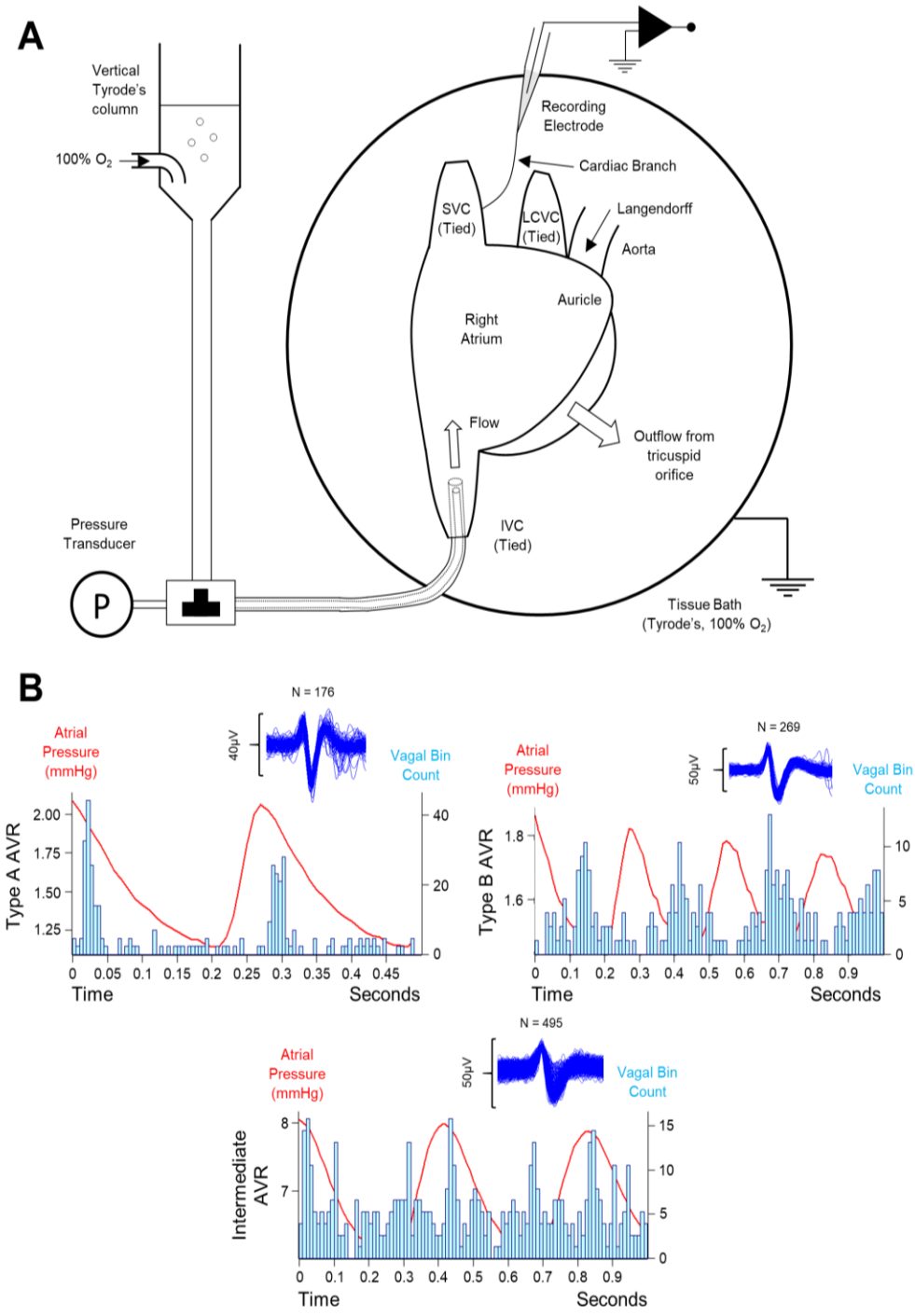

Figure 8: Euthermic model. (A) Schematic illustrating preparation of euthermic heart-vagal nerve model. **(B)** Four examples of type A, type B and intermediate atrial volume receptors recorded using the euthermic model. Shown are overlays of post-stimulus histograms for vagal discharge and mean atrial pressure waveform (red). For type A AVRs, peak discharge occurs during atrial systole. For type B AVRs, peak discharge occur during atrial diastole. For the intermediate AVR, there are two discrete periods of peak discharge: one during atrial systole and one during atrial diastole. Inset are spike overlays (blue) for each recording, indicating single unit recording. Data recorded using Spike2 (CED). AVR, Atrial Volume Receptor. CED, Cambridge Electronic Design. IVC, Inferior Vena Cava. LCVC, Left Cranial Vena Cava. SVC, Superior Vena Cava.

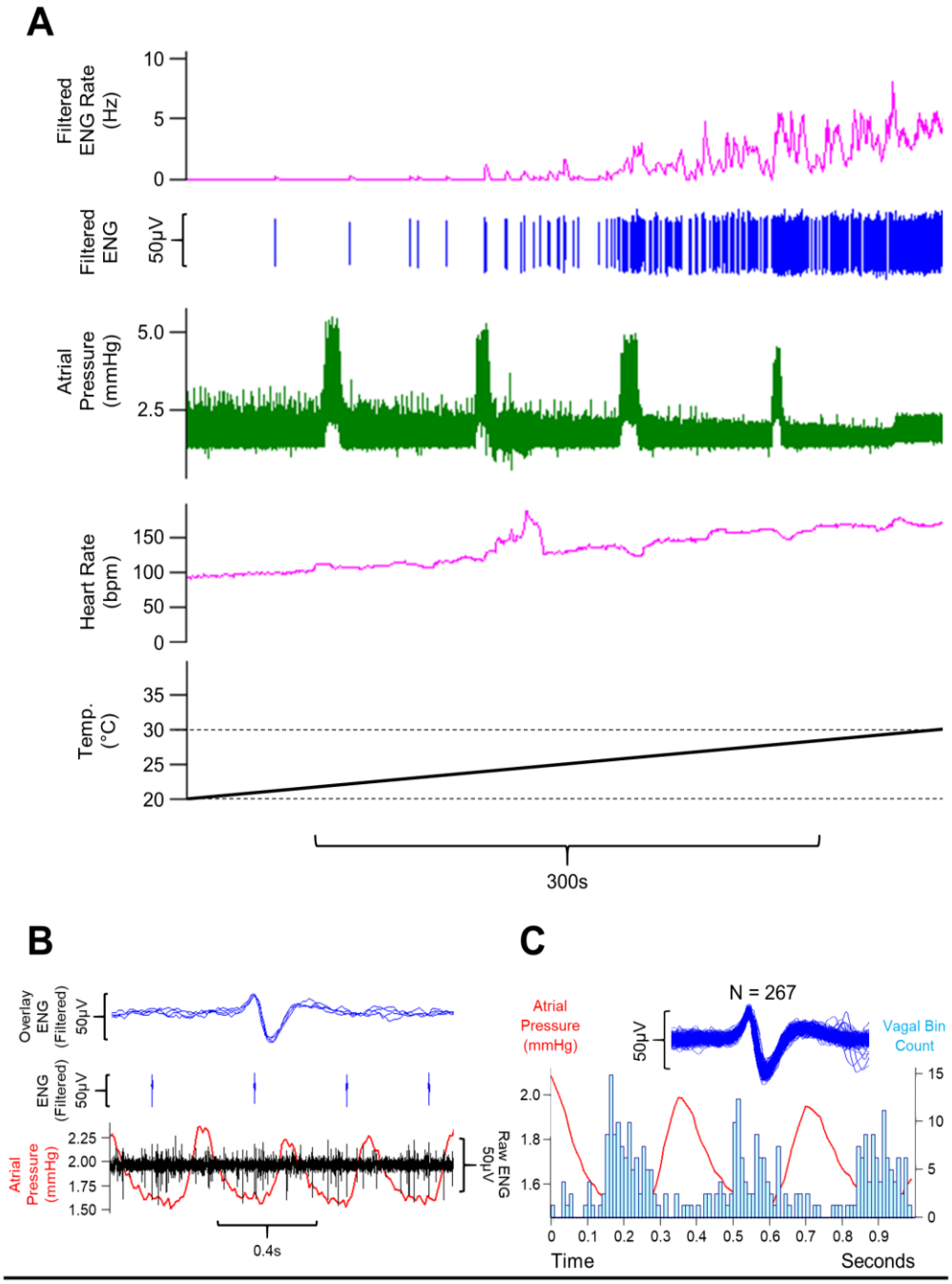

Figure 9: Type B atrial volume receptor activity may be enabled by temperature. (A) Raw data of a single Spike2 (CED) file. Brief periods of increased atrial pressure (green) were applied to elicit discharge from AVRs. Discharge for a single type B AVR (blue) was inactive at room temperature but discharged more frequently as temperature increased (black). Increasing temperature is also reflected by the increase in heart rate and decrease in atrial pressure (negative Treppe phenomenon). **(B)** Zoomed view of the data presented in (A). Overlay of atrial pressure (red) with raw ENG (black) showing multi-unit activity during the atrial cycle. The same single type B AVR (blue) is shown to discharge during atrial diastole. **(C)** Mean atrial pressure waveform (red) overlaid with a post-stimulus histogram of spike activity indicates that the single unit isolated in (A) and (B) discharged predominantly during atrial diastole. Inset is a spike overlay (blue) for this single unit recording. AVR, Atrial Volume Receptor. CED, Cambridge Electronic Design. ENG, Electroneurogram.